Available online at www.sciencedirect.com

SCIENCE DIRECT

Virology 339 (2005) 239 – 248

VIROLOGY

www.elsevier.com/locate/yviro

# Imbalance of tumor necrosis factor receptors during progression in bovine leukemia virus infection

Satoru Konnai[a,*], Tatsufumi Usui[a], Manabu Ikeda[b], Junko Kohara[c], Toh-ichi Hirata[b], Kosuke Okada[b], Kazuhiko Ohashi[a], Misao Onuma[a]

[a]*Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan*
[b]*Faculty of Agriculture, Iwate University, Morioka, Iwate 020-8550, Japan*
[c]*Hokkaido Animal Research Center, Shintoku, Hokkaido 081-0038, Japan*

Received 23 March 2005; returned to author for revision 6 May 2005; accepted 2 June 2005
Available online 1 July 2005

**Abstract**

Previously, we found an up-regulation of tumor necrosis factor alpha (TNF)-α and an imbalance of TNF receptors in sheep experimentally infected with bovine leukemia virus (BLV). In order to investigate the different TNF-α-induced responses, in this study we examined the TNF-α-induced proliferative response and the expression levels of two distinct TNF receptors on peripheral blood mononuclear cells (PBMC) derived from BLV-uninfected cattle and BLV-infected cattle that were aleukemic (AL) or had persistent lymphocytosis (PL). The proliferative response of PBMC isolated from those cattle with PL in the presence of recombinant bovine TNF-α (rTNF-α) was significantly higher than those from AL cattle and uninfected cattle and the cells from PL cattle expressed significantly higher mRNA levels of TNF receptor type II (TNF-RII) than those from AL and BLV-uninfected cattle. No difference was found in TNF-RI mRNA levels. Most cells expressing TNF-RII in PL cattle were $CD5^+$ or $sIgM^+$ cells and these cells showed resistance to TNF-α-induced apoptosis. Additionally, there were significant positive correlations between the changes in provirus load and TNF-RII mRNA levels, and TNF-α-induced proliferation and TNF-RII mRNA levels. These data suggest that imbalance in the expression of TNF receptors could at least in part contribute to the progression of lymphocytosis in BLV infection.


*Keywords:* BLV; TNF receptor; TNF-RII; Proliferation; Apoptosis

## Introduction

Bovine leukemia virus (BLV) is a type C retrovirus which is genetically and structurally similar to human T-lymphotropic virus types (HTLV) I and II (Sagata et al., 1985). Most of the infected cattle do not show any clinical signs of the disease and are referred to as aleukemic (AL) (Burny et al., 1988). Approximately 30% of cattle naturally infected with BLV develop persistent lymphocytosis (PL) with non-malignant polyclonal expansion of $CD5^+$ B-cell, the majority of which harbors BLV provirus (Mirsky et al., 1996). After a latency period of 1–8 years, only 1–5% of the infected cattle develop malignant B-cell lymphosarcoma (LS) (Schwartz and Levy, 1994). Thus, the progression of BLV infection is divided into three stages: AL, PL and LS. Although the pathogenesis of infection clearly involves immunoregulatory host factors, including expression of the cytokines (Kabeya et al., 2001a), the exact mechanism of the disease progression from AL to PL or LS in BLV-infected cattle is not yet known.

A knowledge of which cytokines are related to the disease progression in infected animals is important for understanding the biology of the virus and the pathogenesis of BLV-associated diseases. To date, several cytokines have been reported to be associated with disease progressions of BLV infection, such as interleukin (IL)-2, IL-4, IL-10, IL-12, interferon (IFN)-γ (Keefe et al., 1997a, 1997b, Pyeon

* Corresponding author. Fax: +81 11 706 5217.
*E-mail address:* konnai@vetmed.hokudai.ac.jp (S. Konnai).

0042-6822/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2005.06.010

and Splitter, 1998; Pyeon et al., 1996; Yakobson et al., 1998; Trueblood et al., 1998; Amills et al., 2002; Konnai et al., 2003a) and tumor necrosis factor (TNF)-α (Kabeya et al., 1999, 2001b). In the previous studies, we found that the expression of TNF-α and its receptors closely associated with disease progression in sheep experimentally infected with BLV (Kabeya et al., 1999, 2001b). Interestingly, we found conflicting roles of TNF-α in sheep experimentally infected with BLV. The TNF-α mRNA expression was significantly up-regulated in BLV-resistant sheep; however, down-regulation of TNF-α was found in susceptible sheep (Kabeya et al., 1999). Most recently, Muller and co-workers reported the lack of TNF-α supported persistence of BLV infection in TNF−/− mice (Mullar et al., 2003). These results suggest that in the early phase of infection, TNF-α may contribute to the elimination of BLV. In contrast, TNF-α strongly induced the proliferative response of PBMC in sheep with higher level of BLV in late phase of infection, and mRNA expression of TNF receptor type I (TNF-RI) of these sheep was down-regulated compared to BLV-negative sheep (Kabeya et al., 2001b). These finding suggest the imbalance of TNF receptors contribute to the progression of the disease in BLV-infected animals. However, the mechanism for such proliferation and the phenotypes of the cells expressing the TNF receptors remained obscure.

TNF-α is a multifunctional cytokine produced by several kinds of cells and its activity is mediated by two functionally different cell surface receptors, TNF-RI (55 kDa) and TNF-RII (75 kDa) (Hohmann et al., 1989). Both TNF-RI and -RII are co-expressed in several different tissues and the conflicting activities of TNF-α, including induction and suppression of apoptosis, are thought to be triggered via these two receptors. Most biologic responses of TNF-α such as induction of cell death via apoptosis, cytokine production and cytotoxicity are mediated by TNF-RI. However, recent data have demonstrated that TNF-RII is able to mediate TNF biological activity independently (Grell et al., 1998; Haridas et al., 1998). Due to the lack of intracellular death domain, TNF-RII is involved in the proliferation of thymocytes and the murine cytotoxic T-cell line, and in the TNF-dependent proliferative response of human mononuclear cells (Gehr et al., 1992; Grell et al., 1998; Tartaglia and Goeddel, 1992; Tartaglia et al., 1991; Vandenabeele et al., 1992). TNF-RII signaling appears to be mainly confined to cells of the immune system that makes it of high relevance in understanding immune processes involved in viral infections. Both receptors can bind to TNF with high affinity, but the exact role of the two receptors in mediating the effects of TNF and viral infections is still debated (Herbein and O'Brien, 2000). Several investigations have revealed the relationships between TNF or its receptors and several diseases. The change in the expression patterns of these TNF receptors can determine whether the cells undergo apoptosis or proliferate as in leukemia or lymphoma (Gibbons et al., 1994; Waage et al., 1992). In viral infection, TNF and TNF receptors usually have been considered a main component of antiviral activity, often acting synergistically with IFN-γ. However, in recent years, growing evidence has shown that both DNA and RNA viruses can interfere with the TNF/TNFR pathway to escape immune surveillance (Guidotti and Chisari, 2000; Herbein and O'Brien, 2000). These observations raise the possibility that the differences in TNF receptors expression could result from differences in BLV disease progression, especially the development of LS and PL. However, limited information is available concerning the roles of TNF and its receptors on the progression of disease in BLV-infected animals. In the present experiment, to clarify the role of TNF and its receptors on the disease progression of the BLV infection, we examined the expression of TNF receptors, phenotypes of cells expressing TNF receptors and the ability of TNF-α to induce apoptosis or proliferative response in BLV-infected cattle at two subclinical stages of infection, AL and PL. We showed that imbalance in expression of TNF receptors could result from differences in the disease progression of BLV infection.

## Results

### *Up-regulation of TNF-RII expression in PBMC derived from PL cattle*

In order to investigate the differences in the expression levels of the TNF receptors in BLV-infected cattle at different disease stages, we used real-time PCR to quantify the expression of TNF-RI and TNF-RII mRNAs in fresh PBMC isolated from normal, AL and PL cattle (Fig. 1). In contrast to the previous study of sheep, no difference in the expression levels of TNF-RI mRNA was observed among the different stages of BLV infection in cattle (Fig. 1A). However, the mean expression level of TNF-RII mRNA in PBMC isolated from PL cattle (0.99 ± 0.54) was significantly higher than that of uninfected cattle (0.44 ± 0.24, $P < 0.01$), determined by a one-tailed Student's $t$ test (Fig. 1B). Similarly, the mean expression level of TNF-RII mRNA was significantly increased in AL (0.71 ± 0.54) compared to BLV-negative cattle ($P < 0.05$). The PL stage is characterized by a polyclonal expansion of B lymphocytes co-expressing $CD5^+$ and surface immunoglobulin $M^+$ (sIgM) molecules. To test for differences in the expression protein levels of the TNF receptors, we measured the proportions of TNF receptors expressing cells by flow cytometry. A limited number of cattle were tested, yet as expected, the proportion of $TNF\text{-}RII^+$ cells was clearly increased in PL cattle compared to AL- and BLV-negative cattle (Table 1). Significant expansions of the $CD5^+TNF\text{-}RII^+$ ($P < 0.05$) and $sIgM^+TNF\text{-}RII^+$ cell ($P < 0.001$) population were detected in cattle with PL, but with variable contributions to the overall proliferation of total $TNF\text{-}RII^+$ cells. Concerning the percentages of the $CD4^+TNF\text{-}RII^+$, $CD8^+TNF\text{-}RII^+$, $WC1^+TNF\text{-}RII^+$ and $CD25^+TNF\text{-}RII^+$ cells, each BLV-

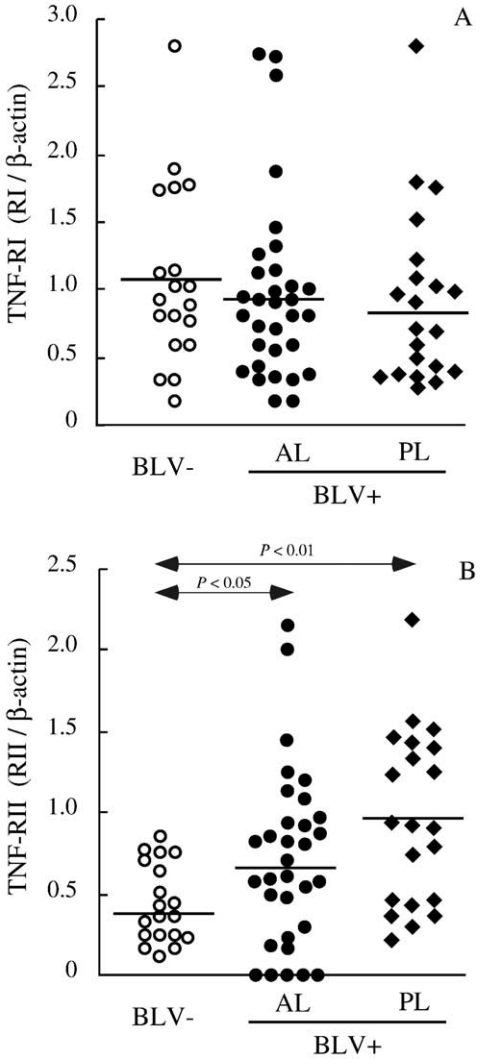


Fig. 1. Quantification of the expression of TNF receptors mRNA (A: RI, B: RII) in PBMC by real-time PCR analysis. Individual dots indicate nineteen BLV-uninfected (empty circle), thirty-two AL (full circles) and twenty-one PL (full diamonds) cattle, respectively. The results were shown as the ratios obtained by dividing the concentrations of the PCR products from the TNF receptors mRNAs by the dose from the β-actin mRNA. Each line in the dots indicates the mean mRNA levels in each group. Differences between groups were considered significant if probability values of $P < 0.05$ were obtained.

infected cattle and control cattle showed a similar distribution, but numbers of these cells were also significantly increased between PL cattle and controls. In contrast to TNF-RII protein levels, no difference in the expression levels of TNF-RI was observed among the stages of BLV infection (data not shown). The results obtained from the flow cytometric analysis were consistent with the results from real-time PCR for the quantification of TNF-RII mRNA in the PBMC mentioned above.

*Provirus loads and TNF-RII expression*

To evaluate the association of the TNF-RII expression with viral propagation in BLV-infected individuals, the provirus loads were determined in BLV-infected cattle with PL and AL by real-time PCR system. The provirus load was significantly increased in the cattle with PL (18.6 ± 12.1%) compared to cattle with AL (7.9 ± 10.0%, $P < 0.0001$). To determine if this increased expression of TNF-RII mRNA correlated to provirus loads, we statistically analyzed with Pearson correlation coefficients (Fig. 2). Significant positive correlations between the provirus loads and TNF-RII mRNA levels were detected ($r = 0.425229$, $P < 0.01$). Thus, this result suggests that up-regulation of TNF-RII expression could influence the expansion of BLV-infected cells during the disease progression from AL to PL. To check this possibility, we further examined if the expression of TNF-RII is specifically up-regulated in BLV-infected cells by investigating the proportions of TNF-RII$^+$p24$^+$ cells in fresh PBMC by flow cytometry. However, BLV-p24$^+$ cells were at low detectable levels in the PBMC freshly isolated from PL cattle (data not shown). In addition, to analyze the association between TNF-RII expression and BLV regulatory gene product, Tax, we investigated the Tax mRNA expression in cattle that were staged as AL or PL. Tax plays an important role in BLV and HTLV-I pathogenesis because Tax is thought to up-regulate several cellular genes including cytokines and their receptors. However, no detectable expression of BLV Tax was observed in fresh PBMC containing cells expressing high levels of TNF-RII when RNA transcription of the BLV-tax gene was analyzed (data not shown).

*Resistance to TNF-α-induced apoptotic death in PBMC from PL cattle*

To evaluate whether BLV infection can modulate the susceptibility of PBMC to TNF-α-induced apoptosis, we examined the percentages of specific apoptosis by bovine rTNF-α in PBMC derived from cattle with AL-, PL- and BLV-uninfected cattle. As shown in Fig. 3, PBMC from AL (54.5 ± 15.2%) and control (63.7 ± 13.1%) cattle were susceptible to TNF-α-induced apoptosis. In contrast, the mean percentage of specific apoptosis in PL cattle (25.0 ± 17.8%) was significantly lower than that in AL ($P < 0.001$)- or BLV-negative cattle ($P < 0.001$). A combination of both rIL-2 and rTNF-α allowed a synergistic effect and reduced the level of apoptosis in PL cattle (9.5 ± 13.2%), although there were no marked effect in control (60.8 ± 14.1%) and AL (47.3 ± 17.0%) cattle. The mean of specific apoptosis was significantly reduced in the combination of both rIL-2 and rTNF-α compared to rTNF-α alone in PBMC from PL cattle ($P < 0.05$). The results show that the TNF-RII high expressing PBMC from PL cattle are resistant to TNF-α-induced apoptosis and the reaction is enhanced by presence of IL-2.

*High proliferative responses to bovine rTNF-α in PBMC from PL cattle*

To test if differences in the expression levels of the TNF receptors contribute to the TNF-α-induced proliferative

Table 1
Subpopulations of TNF-RII-expressing cells in control and BLV-infected cattle with AL and PL

| Disease stage[a] | No. of cattle | No. of lymphocyte (/mm$^3$) | RII/RI ratio | No. (%) of lymphocyte (/mm$^3$) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | CD4$^+$ RII$^+$ | CD8$^+$ RII$^+$ | WC1$^+$ RII$^+$ | CD25$^+$ RII$^+$ | CD5$^+$ RII$^+$ | sIgM$^+$ RII$^+$ |
| Controls | 8 | 3661 ± 1030 | 1.2 ± 0.3 | 117 ± 174 (4.4 ± 2.5) | 124 ± 32 (3.5 ± 0.6) | 145 ± 50 (4.2 ± 1.7) | 170 ± 64 (5.3 ± 2.1) | 41 ± 10 (1.2 ± 0.4) | 55 ± 26 (1.7 ± 1.2) |
| AL | 13 | 4728 ± 1063 | 1.2 ± 0.4 | 280 ± 204* (5.8 ± 4.1) | 192 ± 69 (4.2 ± 1.4) | 155 ± 79 (3.3 ± 1.6) | 356 ± 320 (6.1 ± 2.7) | 82 ± 38** (1.7 ± 0.7) | 42 ± 20 (0.9 ± 0.3) |
| PL | 11 | 14,282 ± 2608 | 1.9 ± 1.2 | 729 ± 626** (5.8 ± 4.2) | 532 ± 248** (4.8 ± 3.8) | 681 ± 678* (6.0 ± 4.7) | 1058 ± 711*** (8.5 ± 4.9) | 2229 ± 2322* (18.0 ± 17.1) | 2010 ± 1394*** (16.4 ± 11.7) |

[a] Animals were regarded as positive for PL if at least two consecutive lymphocyte counts was more than 10,000/mm$^3$.
* $P < 0.05$; comparisons are between BLV-infected cattle and control cattle and were established with a Student's $t$ test.
** $P < 0.01$; comparisons are between BLV-infected cattle and control cattle and were established with a Student's $t$ test.
*** $P < 0.001$; comparisons are between BLV-infected cattle and control cattle and were established with a Student's $t$ test.

response in BLV-infected cattle at different disease stages, we examined the proliferative response to bovine rTNF-α in PBMC derived from cattle with AL-, PL- and BLV-uninfected cattle (Table 2). The means proliferative responses to rTNF-α were higher in BLV-infected cattle compared to BLV-negative cattle, although there were some individual exceptions. The mean stimulation index (SI) of proliferative responses to rTNF-α in cells from BLV-negative cattle was approximately 2.3, and this mean was five times higher in PL cattle (SI = 10.8). The mean proliferative response in PL cattle was significantly higher than that in AL (SI = 6.4, $P < 0.05$) or BLV-negative cattle ($P < 0.01$). Similarly, the mean proliferative response to rTNF-α was significantly increased in AL- compared to BLV-negative cattle ($P < 0.05$, Fig. 4A). To confirm the ability of cell proliferation, we assayed with several mitogens, namely, LPS, Con A and PWM (Table 2). Although the mean response with LPS was no significant difference, response to Con A and PWM were higher in BLV-negative cattle than those of BLV-infected cattle. There was also no significant difference in the response to mitogens between AL and PL cattle. Thus, these results suggest that the ability of cells to proliferate in response to TNF-α increases in BLV-infected cattle. To determine if this increased expression of TNF-RII mRNA correlated to TNF-dependent proliferation, we statistically analyzed with Pearson correlation coefficients (Fig. 4B). Significant positive correlations between the TNF-α-induced proliferation and TNF-RII mRNA levels were detected ($r$ = 0.474153, $P < 0.0001$). Thus, these results suggest that up-regulation of TNF-RII expression could influence the proliferation of cells during disease progression, such as PL.

## Discussion

TNF has a unique role in regulating the immune response to viral infections after its binding to the specific receptors, TNF-RI and TNF-RII, and the susceptibility to viral infection and development of the disease was shown to be determined by TNF-induced functions, and linked to the TNF receptors (Guidotti and Chisari, 2000; Herbein and O'Brien, 2000). In BLV-infected sheep, a good correlation was observed between up-regulation of TNF-α and BLV

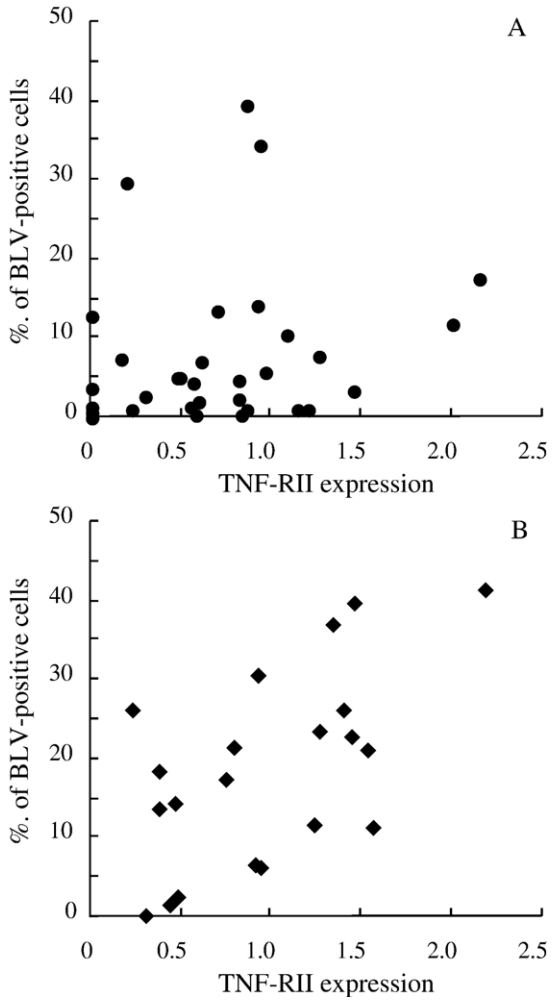


Fig. 2. Correlation of the provirus load and TNF-RII mRNA expression in BLV-infected cattle. The percentages of BLV-positive cells were determined using a system for real-time quantitative PCR. Results of expression levels of TNF-RII mRNA are corresponded to Fig. 1B. Individual dots indicate 32 AL (A) and 21 PL (B) cattle, respectively. Statistical analyses were performed with Pearson correlation coefficients.

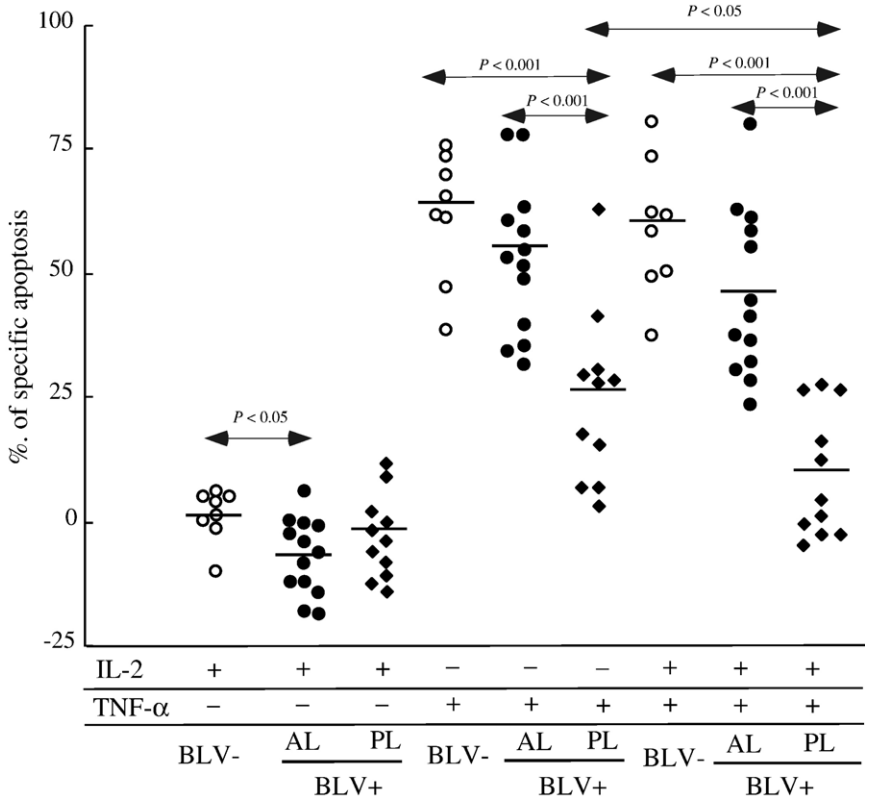


Fig. 3. Susceptibility of PBMC to TNF-α-induced apoptosis in BLV-infected cattle at different disease stages. Individual dots indicate eight BLV-uninfected (empty circle), thirteen AL (full circles) and eleven PL (full diamonds) cattle, respectively. Tested cattle correspond to Table 1. All samples were cultured for 48 h in the presence of human rIL-2 (1 U/ml) or bovine rTNF-α (40 μg/ml) or mixture of rIL-2 and rTNF-α. The cell were stained with 7-AAD and the apoptotic cells were analyzed by flow cytometer. The data represent the percentage of apoptotic cells after each treatment from which background apoptosis (medium only) is subtracted. Line in the dots indicates the mean percentage of specific apoptosis in each group. Differences between groups were considered significant if probability values of $P < 0.05$ were obtained.

clearance (Kabeya et al., 1999). Therefore, we hypothesized that the imbalanced expression of the TNF receptors is responsible for the conflicting TNF-induced responses in BLV infection. In the present study, to evaluate this possibility, we monitored the expression of TNF receptors during the different stages of BLV infection, and found significantly different expression levels of TNF-RII in PBMC from cattle with in the PL stage of BLV infection when compared to those with the AL stage or normal controls. We also found higher anti-apoptosis activity and proliferative responses to rTNF-α in PBMC-expressing TNF-RII isolated from PL cattle than that from uninfected cattle. Con A, a lectin that induces T-cell proliferation by specific interaction with the T-cell receptor complex and a strong inducer of cell proliferation in normal bovine PBMCs, was used as a control for cell proliferative response. Further, PWM, which is a B-cell mitogen, was used as the cell division stimulant. However, our observations indicated that the proliferative responses to these mitogens were lower in PL cattle than in BLV-negative cattle, consistent with the previously reported observations (Ferens and Hovde, 2000). Notwithstanding these observations, we found that TNF-α strongly induced the proliferative response of PBMC in cattle with PL in late phase of infection. These observations raise the possibility that the differences in TNF-α-dependent proliferative responses could result from differences in the expression pattern of TNF receptors.

Although the role of TNF-RII in the tumorigenesis of the BLV-induced PL is still speculative, the TNF receptor superfamily including Fas, CD27, CD30, TNF-RI and TNF-RII is a group of cell surface proteins involved in differentiation, activation, proliferation and apoptosis of cells (Smith et al., 1994). The proliferation of activated peripheral lymphocytes can be enhanced by TNF-α and also with TNF-RII mAb (Tartaglia et al., 1993; Yokota et al., 1988), suggesting that TNF-RII is mainly responsible for the proliferative effects of TNF-α in cells. TNF-receptor-associated factor 2 (TRAF-2) homodimers and TRAF-1-TRAF-2 heterodimers can associate with the C-terminal region in cytoplasmic domain of TNF-RII, which is required for signaling, proliferation and activation of nuclear factor-κB (NF-κB) (Rothe et al., 1994, 1995a). Inhibitor of apoptosis proteins c-IAP1 and c-IAP2 associates with TRAF1 and TRAF2 as part of the TNF-RII signaling complex and can inhibit apoptosis by directly affecting the down proteases caspase-3 and caspase-7 (Rothe et al., 1995b; Roy et al., 1997). Previously, we observed that TNF-α strongly induced

Table 2
PBMC proliferative responses in control and BLV-infected cattle with AL and PL

| Disease stage | No. of cattle | Stimulation index (mean ± SD)[a] | | | |
|---|---|---|---|---|---|
| | | rTNF-α (40 μg/ml) | LPS (25 μg/ml) | Con A (5 μg/ml) | PWM (1 μg/ml) |
| Controls | 20 | 2.3 ± 3.4 | 2.5 ± 2.9 | 341.3 ± 264.9 | 51.6 ± 39.4 |
| AL | 32 | 6.4 ± 5.0* | 2.4 ± 2.3 | 117.7 ± 103.9** | 35.6 ± 37.7 |
| PL | 21 | 10.8 ± 7.7** | 3.7 ± 4.5 | 93.0 ± 99.2** | 27.7 ± 19.4** |

[a] All samples were tested in triplicate, and the stimulation index was defined as mean counts of test samples divided by mean counts of controls.
* $P < 0.05$; comparisons are between BLV-infected cattle and control cattle and were established with a Student's $t$ test.
** $P < 0.01$; comparisons are between BLV-infected cattle and control cattle and were established with a Student's $t$ test.

the proliferative response of PBMC in sheep with high level BLV in the late phase of infection, and then mRNA expression of TNF-RI was down-regulated in PBMC from the sheep with high levels of BLV compared to BLV-negative sheep (Kabeya et al., 2001a, 2001b). In the present experiment, we observed the up-regulation of TNF-RII expression on PBMC from PL cattle, although we could not observe a significant different expression of TNF-RI on PBMC in BLV-infected cattle. The difference was not statistically significant possibly due to the limited numbers of subjects available or because of a variable infectious period. One possible explanation for the enhanced anti-apoptosis activity and high proliferative response to TNF-α in PBMC isolated from PL relates to the different kinetics of signaling by imbalance of TNF receptors, such as down-regulation of TNF-RI or up-regulation of TNF-RII, although the exact mechanism in the regulations of TNF receptors expression is still not clear. Further analysis of activation of NF-κB or protease caspase in relation to the different expression of TNF receptors is required to clarify this point. Our results suggest that the imbalance of TNF receptors could influence the apoptosis and proliferation of cell during the disease progression from aleukemic stage to lymphocytosis.

Interestingly, increased expression of TNF-RII but not TNF-RI have been also reported in a BLV-infected B cell line, KU-1 (Kabeya et al., 2001a, 2001b) and HTLV-I-infected T-cell lines (Yang et al., 2002). Indeed, Yang et al. (2002) reported the HTLV-I-infected cell lines expressing TNF-RII were resistant to TNF-α-mediated apoptosis. In the present experiment, the TNF-RII high expressing PBMC from PL cattle are strongly resistant to TNF-α-induced apoptosis and the reaction is enhanced by presence of IL-2. These findings suggest that similar responses such as resistance to apoptosis of the cells might occur in TNF-RII-expressing cell lines derived from HTLV-I-infected patients and BLV-infected-cell lines. Additionally, at the PL stages, increased population of $CD5^+$ B-cells expressed higher levels of TNF-RII. Trueblood et al. (1998) found B-cell proliferation during BLV-induced PL was enhanced by T-cell-derived IL-2. Although IL-2 may be a major factor for cell proliferation, TNF-α, as shown in the case of the proliferation of PBMC in this study, is also likely to play a role in proliferation on retroviral infections such as BLV or HTLV-I. It has been proposed that the expression of CD25 as IL-2 receptor and IL-2/CD25 autocrine or paracrine loop play a part in maintaining the very high proviral loads often found in BLV infection (Stone et al., 1994, 1995, 1997; Trueblood et al., 1998). Indeed, at the PL stages, increased population of $CD25^+$TNF-$RII^+$ cells was observed in the present experiment. Furthermore, it is possible that the increased expression of TNF-RII may assist the IL-2-induced cell proliferation by anti-apoptosis and contribute to the development of lymphocytosis or lymphoma in BLV-infected animals (Fig. 5).

In HTLV-I infection, aberrant production of several cytokines and their receptors was thought to be a result of the transactivating ability of the Tax protein of HTLV-I (Uchiyama, 1997; Leung and Nabel, 1988; Ballard et al., 1988; Nakajima et al., 1993). These observations raise the possibility that the up-regulation of TNF-RII expression might be mediated by transactivation through the BLV regulatory gene product, Tax. To check this possibility, the RNA transcript of BLV-tax gene was analyzed in TNF-RII high expressing cell. However, no detectable expression of BLV Tax was observed in fresh PBMC containing cells expressing high levels of TNF-RII when RNA transcription of the BLV-tax gene was analyzed (data not shown). In addition, we failed to confirm the specific expression of TNF-RII on BLVp24-expressing cells, although more studies need to be conducted (data not shown). At present, it is difficult to determine whether the expression of TNF-RII is specifically up-regulated via virus expansion after infection by BLV, despite its clear effect on the provirus load. As alternative expression for the up-regulation of TNF-RII, the mechanism by which up-regulation of TNF-RII expression might be mediated by IFN-γ. Interestingly, the expression of TNF-RII but not of TNF-RI was up-regulated significantly after IFN-γ treatment, and rescues TNF-α-induced apoptosis (Horie et al., 1999). IFN-γ is of central importance in viral dynamics because, along with other cellular immunity-associated cytokines, it is thought to be a protective factor in the progression of BLV infection. Indeed, up-regulation of IFN-γ mRNA has been reported after infection of BLV (Keefe et al., 1997a, 1997b; Yakobson et al., 1998; Konnai et al., 2003a). Although this is in contrast to the previous reports that IFN-γ is effective in viral elimination, indirect effects of IFN-γ may assist TNF-α-induced proliferation via TNF-RII. The mechanism of the up-regulation of TNF-RII during the disease progression from AL to PL, however, remains unknown.

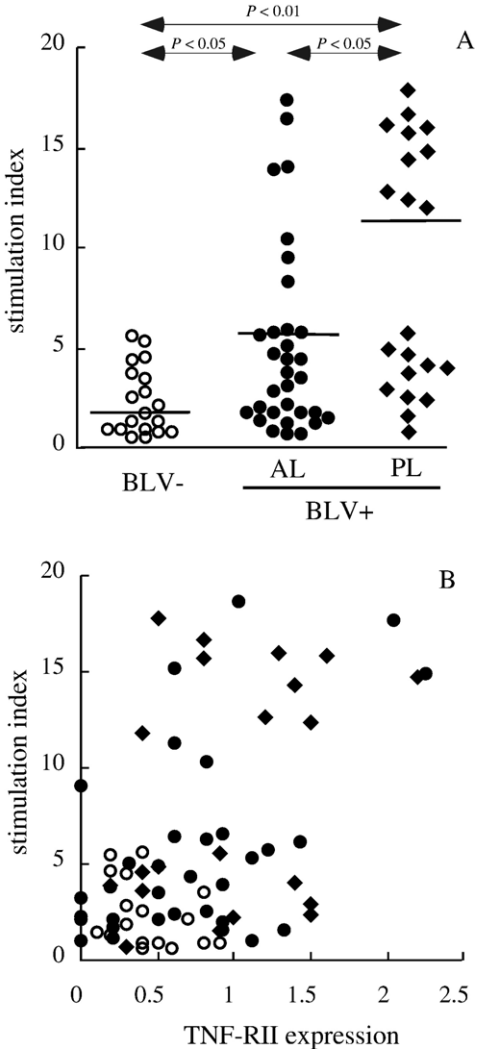


Fig. 4. (A) Proliferative responses of PBMC to recombinant bovine TNF-α in BLV-infected cattle at different disease stages. Individual dots indicate nineteen BLV-uninfected (empty circle), thirty-two AL (full circles) and twenty-one PL (full diamonds) cattle, respectively. All samples were tested in triplicate. The data were presented as mean stimulation index (SI: defined as mean counts of test sample divided by mean counts of control) in each of cattle. Line in dots indicates the mean SI in each group. Differences between groups were considered significant if probability values of $P < 0.05$ were obtained. (B) Correlation of the TNF-RII mRNA expression and proliferative response to bovine rTNF-α. Results of expression levels of TNF-RII mRNA and rTNF-α-induced proliferation correspond to Figs. 1A and 4A. Statistical analyses were performed with Pearson correlation coefficients.

In conclusion, we present here an aberrant expression of TNF-RII, anti-apoptotic activity and a high proliferative response of PBMC to TNF-α in PL cattle. TNF-α-induced proliferative response via anti-apoptosis through its binding to TNF-RII might contribute to the progression of bovine leukosis in animals which develop persistent lymphocytosis of B cells. It is possible that TNF-RII is at least one of the proliferative-associated molecules during BLV infection since lymphocytes from the PL stages of cattle expressed higher levels of TNF-RII as seen in chronic lymphocytic leukemia (Trentin et al., 1994; Waage et al., 1992), Epstein–Barr virus-transformed B cells and Burkitt lymphoma (Gibbons et al., 1994) and HTLV-I infection (Yang et al., 2002). Further studies are required to clarify the mechanism in more detail to understand why the different expressions of TNF receptors were observed in BLV-induced disease progressions and to determine whether the expression of TNF-RII is specifically up-regulated on BLV-infected cells.

## Materials and methods

### *Animals and BLV detections*

The cattle were maintained within the Iwate university-associated farm or the animal research center of Hokkaido dairy herd research herd, and all were naturally infected with BLV. BLV infection was tested by an agar gel immunodiffusion test with gp51 as antigen (Onuma et al., 1975) and was further confirmed by PCR with a primer pair (BLV-LTR1; 5′-TAA TAC GAC TCA CTA TAT AGG G-3′ and BLV-LTR533; 5′-GCC GCT CTA GAA CTA GTG GAT CCC-3′) to amplify the BLV long terminal region (LTR) using a thermal cycler (System 9700, Applied Biosystems) as described previously (Tajima et al., 1998). BLV-positive animals were classified as AL or PL according to numbers of leukocytes and percentage of lymphocytes in whole blood (Thurmond et al., 1990). Animals were regarded as PL if at least two consecutive leukocyte counts including lymphoid cells were more than 10,000/μl. Percentage of BLV-infected cells was calculated from genomic DNA using real-time PCR as described previously (Tajima et al., 2003). Real-time PCR using SYBER Green I was performed using a LightCycler™ (Roche Diagnostics) according to the manufacturer's instructions. Following genomic DNA extraction from PBMC with a Wizard DNA purification kit (Promega) as previously described (Konnai et al., 2003b), DNA templates were suspended in 20 μl (total volume) of PCR buffer, to which had been added

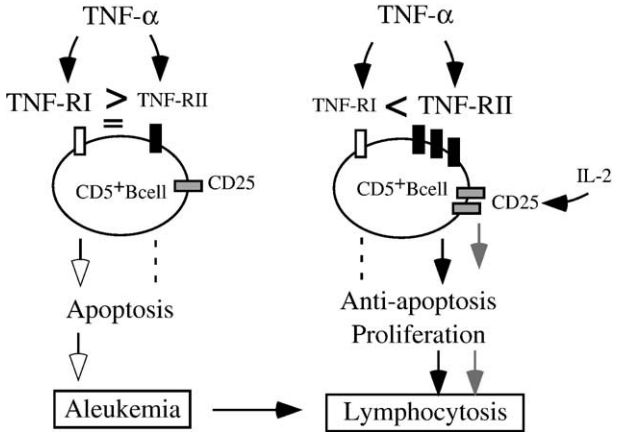


Fig. 5. The possible involvement of TNF receptors in the course of BLV infection.

oligonucleotide primers (final concentration of 0.2 μM; BLV-LTR primer, 256; 5′-GAG CTC TCT TGC TCC CGA GAC-3′ and BLV-LTR453; 5′-GAA ACA AAC GCG GGT GCA AGC CAG-3′ or β-globin primer as control, PC03; 5′-ACA CAA CTG TGT TCA CTA GC-3′ and PC04; 5′-CAA CTT CAT CCA CGT TCA CC-3′), and 2 μl of LightCycler-DNA Master SYBER Green I (Roche Diagnostics). Additionally, to determine whether Tax, a transcriptional activator encoded by the X-region of BLV, was expressed, RT-PCR was performed using RNA samples prepared from fresh PBMC from BLV-infected cattle as described previously (Tajima et al., 2003). Total RNA was extracted from the freshly separated PBMC by using the TRIZOLTM reagent according to the manufacturer's protocol (Invitrogen). All samples were treated with deoxyribonuclease I (amplification grade, Invitrogen) to eliminate DNA contamination. The reverse transcriptase (RT) reaction was performed with 5 μg of purified total RNA. BLV tax and cellular β-actin cDNA were amplified by PCR using thermal cycler with primers BLV tax/rex 4758 (5′-AGG CGC TCT CCT GGC TAC TG-3′) and BLV tax/rex 7333 (5′-GGC ACC AGG CAT CGA TGG TG-3′) and boβ-actin F (5′-CGC ACC ACT GGC ATT GTC AT-3′) and boβ-actin R (5′-TCC AAG GCG ACG TAG CAG AG-3′), respectively.

*Quantitation of TNF receptor mRNAs by real-time PCR*

Total RNA extraction from the freshly separated PBMC, cDNA synthesis and real-time quantitative RT-PCR were performed as described previously (Konnai et al., 2003a). The cDNA template was mixed with a total volume of 20 μl of PCR buffer with oligonucleotide primers (0.2 mM each, final conc.), either boTNF-RI F (5′-CGC CTC TGT CGT CTT AGC AT-3′) and boTNF-RI R (5′-TGG AGA CAG GAC TGG AAC TT-3′), or boTNF-RII F (5′-CTC GAC CAG CAG CAC GGA CA-3′) and boTNF-RII R (5′-GCG TCT GTG TCC CTC GTG GA-3′), and 2 μl of SYBER Green I (Roche Diagnostics) was added. We also amplified the bovine β-actin gene in each sample, using a primer pair, boβ-actin F (5′-CGC ACC ACT GGC ATT GTC AT-3′) and boβ-actin R (5′-TCC AAG GCG ACG TAG CAG AG-3′) to monitor the concentrations of template cDNAs.

*Flow cytometry analysis*

To analyze the cells expressing TNF receptors, single- and dual-color flow cytometric analysis was performed using the following antibodies: IL-A11 (mouse anti-bovine CD4), CACT105A (mouse anti-bovine CD5, VMRD), IL-A51 (mouse anti-bovine CD8), IL-A29 (mouse anti-bovine WC1), IL-A111 (mouse anti-bovine CD25; IL-2R), IL-A30 (mouse anti-bovine surface IgM), H-202 (rabbit anti-human TNF-RI; Santa Cruz Biotechnology), H-202 (rabbit anti-human TNF-RII; Santa Cruz Biotechnology) and BLV3 (mouse anti-BLV p24, VMRD) as described previously (Dequiedt et al., 1997; Konnai et al., 2003c). Purified PBMC ($1 \times 10^7$ cells/ml) were incubated with the optimal concentration of each antibody for 30 min at 4 °C. Then, the cells were washed with sterile PBS containing EDTA (0.75 mg/ml) and labeled with isotype-specific secondary antibodies, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulins (Immunotech) or phycoerythrin (PE)-conjugated goat anti-mouse immunoglobulins (Immunotech). Fluorescence of the cells was measured on an EPICS XL, and the data were analyzed using the SYSTEM IITM software (Beckman Coulter).

*Induction of apoptosis and detection of apoptotic cells*

Since the percentage of apoptotic cell in PBMC ($5 \times 10^5$/ml) from healthy cattle by bovine recombinant TNF-α (40 μg/ml, rTNF-α; a gift from Dr. Yuichi Yokomizo, National Institute for Animal Health, Tsukuba, Japan) in 48-h culture was dose dependent and plateaued at 50 μg/ml, the PBMC derived from cattle with PL and AL or controls were cultured under a concentration of 40 μg/ml. To further investigate the combined effects of IL-2 that were thought to be a major proliferative factor in BLV infection, cells were incubated with the optimal concentration of human recombinant IL-2 (rIL-2, 1U/ml, Wako Chemicals) which was added simultaneously as described previously (Trueblood et al., 1998). Apoptosis was measured after 48 h cultivation with various treatments. To measure apoptosis, we used the DNA dye 7-aminoactinomycine D (7-AAD) as described previously (Yang et al., 2002). Briefly, after cell surface labeling, cells were incubated with 7-AAD (20 μg/ml) (Immunotech) in PBS in the dark for 20 min at room temperature, washed twice in PBS and fixed in 1% paraformaldehyde solution containing actinomycin D (AD) (20 μg/ml). The samples were submitted for flow cytometry analysis in a paraformaldehyde AD solution. Ten thousand events were acquired, and apoptotic cells were analyzed using EPICS XL with SYSTEM IITM as above. The apoptotic cells were calculated as described previously (Yang et al., 2002). % specific apoptosis = (% 7-AAD$^+$ cells in the medium containing rTNF-α − spontaneous 7-AAD$^+$ cells in the medium alone) / (100 − % spontaneous 7-AAD$^+$ cells in the medium alone) × 100.

*Proliferation assay*

In order to investigate the influence of TNF-α on TNF-receptor-expressing cell proliferation, we monitored proliferative response to bovine rTNF-α. PBMC ($10^5$ cells/well in 96-well flat-bottom) isolated from PL, AL and control animals were cultured at 37 °C for 72 h with 5% CO2 in the presence of rTNF-α (40 μg/ml), lipopolysaccharide (LPS; 50 μg/ml, Sigma), concanavalin A (Con A; 5 μg/ml, Sigma) and pokeweed mitogen (PWM; 1 μg/ml, Gibco) in a final volume of 200 μl as described previously (Kabeya et al., 2001b). [$^3$H]-Thymidine (0.5 μCi/well) was added to the culture and cultured for an additional 6 h, then the cells were

harvested onto glass filters and incorporated radioactivity was measured by liquid scintillation counter. All samples were tested in triplicate, and the data were presented as mean stimulation index (SI: defined as mean counts of test samples divided by mean counts of controls).

*Statistical analysis*

Data were analyzed by one-way analysis of variance followed by Student's *t* test. Differences between groups were considered significant if probability values of $P < 0.05$ were obtained. To evaluate the relationships between provirus loads and TNF-RII mRNA levels, and TNF-α-induced proliferation and TNF-RII mRNA levels, Pearson correlation coefficients were calculated as described previously (Waldvogel et al., 2000). All statistical analyses were performed with the statistical software StatceL (OMS, Saitama, Japan).

## Acknowledgments

We are indebted to Dr. Yuichi Yokomizo, National Institute of Animal Health, for donating the recombinant bovine TNF-α. We thank Dr. Hidenori Kabeya for critical reading of the article and helpful discussion and Dr. Kinberly Orr for help in preparing the manuscript. This work was partly supported by Recombinant Cytokine's Projects from the Ministry of Agriculture, Forestry and Fisheries, Japan (RCP-1997-2330).

## References

Amills, M., Ramiya, V., Norimine, J., Olmstead, C.A., Lewin, H.A., 2002. Reduced IL-2 and IL-4 mRNA expression in $CD4^+$ T cells from bovine leukemia virus-infected cows with persistent lymphocytosis. Virology 304, 1–9.

Ballard, D.W., Bohnlein, E., Lowenthal, J.W., Wano, Y., Franza, B.R., Greene, W.C., 1988. HTLV-I tax induces cellular proteins that activate the kappa B element in the IL-2 receptor alpha gene. Science 241, 1652–1655.

Burny, A., Cleuter, Y., Kettmann, R., Mammerickx, M., Marbaix, G., Portetelle, D., van den Broeke, A., Willems, L., Thomas, R., 1988. Bovine leukaemia: facts and hypotheses derived from the study of an infectious cancer. Vet. Microbiol. 17, 197–218.

Dequiedt, F., Hanon, E., Kerkhofs, P., Pastoret, P.P., Portetelle, D., Burny, A., Kettmann, R., Willems, L., 1997. Both wild-type and strongly attenuated bovine leukemia viruses protect peripheral blood mononuclear cells from apoptosis. J. Virol. 71, 630–639.

Ferens, W.A., Hovde, C.J., 2000. Antiviral activity of shiga toxin 1: suppression of bovine leukemia virus-related spontaneous lymphocyte proliferation. Infect. Immun. 68, 4462–4469.

Gehr, G., Gentz, R., Brockhaus, M., Loetscher, H., Lesslauer, W., 1992. Both tumor necrosis factor receptor types mediate proliferative signals in human mononuclear cell activation. J. Immunol. 149, 911–917.

Gibbons, D.L., Rowe, M., Cope, A.P., Feldmann, M., Brennan, F.M., 1994. Lymphotoxin acts as an autocrine growth factor for Epstein–Barr virus-transformed B cells and differentiated Burkitt lymphoma cell lines. Eur. J. Immunol. 24, 1879–1885.

Grell, M., Becke, F.M., Wajant, H., Mannel, D.N., Scheurich, P., 1998. TNF receptor type 2 mediates thymocyte proliferation independently of TNF receptor type 1. Eur. J. Immunol. 28, 257–263.

Guidotti, L.G., Chisari, F.V., 2000. Cytokine-mediated control of viral infections. Virology 273, 221–227.

Haridas, V., Darnay, B.G., Natarajan, K., Heller, R., Aggarwal, B.B., 1998. Overexpression of the p80 TNF receptor leads to TNF-dependent apoptosis, nuclear factor-B activation, and c-Jun kinase activation. J. Immunol. 160, 3152–3162.

Herbein, G., O'Brien, W.A., 2000. Tumor necrosis factor (TNF)-alpha and TNF receptors in viral pathogenesis. Proc. Soc. Exp. Biol. Med. 223, 241–257.

Hohmann, H.P., Remy, R., Brockhaus, M., van Loon, A.P., 1989. Two different cell types have different major receptors for human tumor necrosis factor (TNF alpha). J. Biol. Chem. 264, 14927–14934.

Horie, T., Dobashi, K., Iizuka, K., Yoshii, A., Shimizu, Y., Nakazawa, T., Mori, M., 1999. Interferon-gamma rescues TNF-alpha-induced apoptosis mediated by up-regulation of TNFR2 on EoL-1 cells. Exp. Hematol. 27, 512–519.

Kabeya, H., Ohashi, K., Oyunbileg, N., Nagaoka, Y., Aida, Y., Sugimoto, C., Yokomizo, Y., Onuma, M., 1999. Up-regulation of tumor necrosis factor alpha mRNA is associated with bovine-leukemia virus (BLV) elimination in the early phase of infection. Vet. Immunol. Immunopathol. 68, 255–265.

Kabeya, H., Ohashi, K., Onuma, M., 2001a. Host immune responses in the course of bovine leukemia virus infection. J. Vet. Med. Sci. 61, 475–480.

Kabeya, H., Fukuda, A., Ohashi, K., Sugimoto, C., Onuma, M., 2001b. Tumor necrosis factor alpha and its receptors in experimentally bovine leukemia virus-infected sheep. Vet. Immunol. Immunopathol. 81, 129–139.

Keefe, R.G., Choi, Y., Ferrick, D.A., Stott, J.L., 1997a. Bovine cytokine expression during different phases of bovine leukemia virus infection. Vet. Immunol. Immunopathol. 56, 39–51.

Keefe, R.G., Ferrick, D.A., Stott, J.L., 1997b. Cytokine transcription in lymph nodes of cattle in different stages of bovine leukemia virus infection. Vet. Immunol. Immunopathol. 59, 271–283.

Konnai, S., Usui, T., Ohashi, K., Onuma, M., 2003a. The rapid quantitative analysis of bovine cytokine genes by real-time RT-PCR. Vet. Microbiol. 43, 223–232.

Konnai, S., Nagaoka, Y., Takeshima, S., Onuma, M., Aida, Y., 2003b. Sequences and diversity of 17 new Ovar-DRB1 alleles from three breeds of sheep. Eur. J. Immunogenet. 30, 275–282.

Konnai, S., Takeshima, S., Tajima, S., Yin, S.A., Okada, K., Onuma, M., Aida, Y., 2003c. The influence of ovine MHC class II DRB1 alleles on immune response in bovine leukemia virus infection. Microbiol. Immunol. 47, 223–232.

Leung, K., Nabel, G.J., 1988. HTLV-1 transactivator induces interleukin-2 receptor expression through an NF-kappa B-like factor. Nature 333, 776–778.

Mirsky, M.L., Olmstead, C.A., Da, Y., Lewin, H.A., 1996. The prevalence of proviral bovine leukemia virus in peripheral blood mononuclear cells at two subclinical stages of infection. J. Virol. 70, 2178–2183.

Mullar, C., Coffey, T.J., Koss, M., Teifke, J.P., Langhans, W., Werling, D., 2003. Lack of TNF alpha supports persistence of a plasmid encoding the bovine leukemia virus in TNF−/− mice. Vet. Immunol. Immunopathol. 92, 15–22.

Nakajima, T., Kitamura, K., Yamashita, N., Sakane, T., Mizushima, Y., Delespesse, G., Lal, R.B., 1993. Constitutive expression and production of tumor necrosis factor-beta in T-cell lines infected with HTLV-I and HTLV-II. Biochem. Biophys. Res. Commun. 191, 371–377.

Onuma, M., Olson, C., Baumgartener, L.E., Pearson, L.D., 1975. An ether-sensitive antigen associated with bovine leukemia virus infection. J. Natl. Cancer Inst. 55, 1155–1158.

Pyeon, D., Splitter, G.A., 1998. Interleukin-12 p40 mRNA expression in bovine leukemia virus-infected animals: increase in alymphocytosis but decrease in persistent lymphocytosis. J. Virol. 72, 6917–6921.

Pyeon, D., O'Reilly, K.L., Splitter, G.A., 1996. Increased interleukin-10 mRNA expression in tumor-bearing or persistently lymphocytotic animals infected with bovine leukemia virus. J. Virol. 70, 5706–5710.

Rothe, M., Wong, S.C., Henzel, W.J., Goeddel, D.V., 1994. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. Cell 78, 681–692.

Rothe, M., Sarma, V., Dixit, V.M., Goeddel, D.V., 1995a. TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. Science 269, 1424–1427.

Rothe, M., Pan, M.G., Henzel, W.J., Ayres, T.M., Goeddel, D.V., 1995b. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell 83, 1243–1252.

Roy, N., Deveraux, Q.L., Takahashi, R., Salvesen, G.S., Reed, J.C., 1997. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO. J. 16, 6914–6925.

Sagata, N., Yasunaga, T., Tsuzuku-Kawamura, J., Ohishi, K., Ogawa, Y., Ikawa, Y., 1985. Complete nucleotide sequence of the genome of bovine leukemia virus: its evolutionary relationship to other retroviruses. Proc. Natl. Acad. Sci. U.S.A. 82, 677–681.

Schwartz, I., Levy, D., 1994. Pathobiology of bovine leukemia virus. Vet. Res. 25, 521–536.

Smith, C.A., Farrah, T., Goodwin, R.G., 1994. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell 76, 959–962.

Stone, D.M., McElwain, T.F., Davis, W.C., 1994. Enhanced B-lymphocyte expression of IL-2R alpha associated with T lymphocytosis in BLV-infected persistently lymphocytotic cows. Leukemia 8, 1057–1061.

Stone, D.M., Hof, A.J., Davis, W.C., 1995. Up-regulation of IL-2 receptor alpha and MHC class II expression on lymphocyte subpopulations from bovine leukemia virus infected lymphocytotic cows. Vet. Immunol. Immunopathol. 48, 65–76.

Stone, D.M., Norton, L.K., Davis, W.C., 1997. Modulation of bovine leukemia virus-associated spontaneous lymphocyte proliferation by monoclonal antibodies to lymphocyte surface molecules. Clin. Immunol. Immunopathol. 83, 156–164.

Tajima, S., Ikawa, Y., Aida, Y., 1998. Complete bovine leukemia virus (BLV) provirus is conserved in BLV-infected cattle throughout the course of B-cell lymphosarcoma development. J. Virol. 72, 7569–7576.

Tajima, S., Takahashi, M., Takeshima, S., Konnai, S., Yin, S.A., Watarai, S., Tanaka, Y., Onuma, M., Okada, K., Aida, Y., 2003. A mutant form of the tax protein of bovine leukemia virus (BLV), with enhanced transactivation activity, increases expression and propagation of BLV in vitro but not in vivo. J. Virol. 77, 1894–1903.

Tartaglia, L.A., Goeddel, D.V., 1992. Two TNF receptors. Immunol. Today 13, 151–153.

Tartaglia, L.A., Weber, R.F., Figari, I.S., Reynolds, C., Palladino Jr., M.A., Goeddel, D.V., 1991. The two different receptors for tumor necrosis factor mediate distinct cellular responses. Proc. Natl. Acad. Sci. U.S.A. 88, 9292–9296.

Tartaglia, L.A., Goeddel, D.V., Reynolds, C., Figari, I.S., Weber, R.F., Fendly, B.M., Palladino Jr., M.A., 1993. Stimulation of human T-cell proliferation by specific activation of the 75-kDa tumor necrosis factor receptor. J. Immunol. 151, 4637–4641.

Thurmond, M.C., Carter, R.L., Picanso, J.P., Stralka, K., 1990. Upper-normal prediction limits of lymphocyte counts for cattle not infected with bovine leukemia virus. Am. J. Vet. Res. 51, 466–470.

Trentin, L., Zambello, R., Agostini, C., Enthammer, C., Cerutti, A., Adami, F., Zamboni, S., Semenzato, G., 1994. Expression and regulation of tumor necrosis factor, interleukin-2, and hematopoietic growth factor receptors in B-cell chronic lymphocytic leukemia. Blood 84, 4249–4256.

Trueblood, E.S., Brown, W.C., Palmer, G.H., Davis, W.C., Stone, D.M., McElwain, T.F., 1998. B-lymphocyte proliferation during bovine leukemia virus-induced persistent lymphocytosis is enhanced by T-lymphocyte-derived interleukin-2. J. Virol. 72, 3169–3177.

Uchiyama, T., 1997. Human T cell leukemia virus type I (HTLV-I) and human diseases. Annu. Rev. Immunol. 15, 15–37.

Vandenabeele, P., Declercq, W., Van Vercammen, D., de Craen, M., Grooten, J., Loetscher, H., Brockhaus, M., Lesslauer, W., Fiers, W., 1992. Functional characterization of the human tumor necrosis factor receptor p75 in a transfected rat/mouse T-cell hybridoma. J. Exp. Med. 176, 1015–1024.

Waage, A., Liabakk, N., Lien, E., Lamvik, J., Espevik, T., 1992. p55 and p75 tumor necrosis factor receptors in patients with chronic lymphocytic leukemia. Blood 80, 2577–2583.

Waldvogel, A.S., Hediger-Weithaler, B.M., Eicher, R., Zakher, A., Zarlenga, D.S., Gasbarre, L.C., Heussler, V.T., 2000. Interferon-gamma and interleukin-4 mRNA expression by peripheral blood mononuclear cells from pregnant and non-pregnant cattle seropositive for bovine viral diarrhea virus. Vet. Immunol. Immunopathol. 77, 201–212.

Yakobson, B., Brenner, J., Ungar-Waron, H., Trainin, Z., 1998. Short-termed expression of interleukin-12 during experimental BLV infection may direct disease progression to persistent lymphocytosis. Vet. Immunol. Immunopathol. 64, 207–218.

Yang, Y.C., Hsu, T.Y., Lin, R.H., Su, I.J., Chen, J.Y., Yang, C.S., 2002. Resistance to tumor necrosis factor-alpha-induced apoptosis in human T-lymphotropic virus type I infected T cell lines. AIDS Res. Hum. Retrovir. 18, 207–212.

Yokota, S., Geppert, T.D., Lipsky, P.E., 1988. Enhancement of antigen- and mitogen-induced human T lymphocyte proliferation by tumor necrosis factor-alpha. J. Immunol. 140, 531–536.